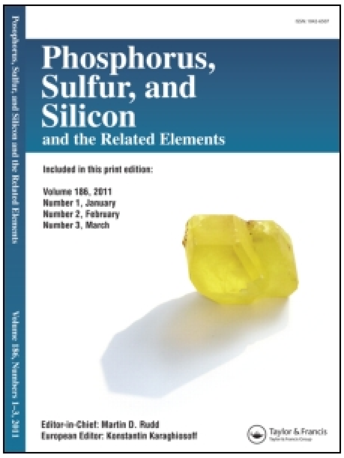

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Cyclic Adenosine 5'-Diphosphate Ribose-Mediated Cellular Signalling: New Insights using Synthetic Ligands

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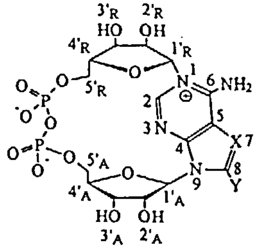
Using a chemoenzymatic approach we synthesized a number of structurally-modified analogues of the novel Ca^{2+}-releasing cyclic nucleotide cADPR. This produced novel agonists, antagonists and enzyme inhibitors to investigate this new signalling pathway, and also the first membrane permeable antagonist.

Keywords: nucleotide; Ca^{2+}-release; cyclic ADP-ribose; cell signalling

Cyclic ADP-ribose (cADPR, 1) is a cyclized ADP ribose having an N-glycosyl linkage between the anomeric carbon of the terminal ribose unit and the N^1-imino group of the adenine ring[1]. It is synthesized from its precursor NAD^+ by ADP-ribosyl cyclases and is hydrolysed by cADPR hydrolases[2,3]. cADPR binds to a specific target on the endoplasmic reticulum[4], where it activates a Ca^{2+} release mechanism with a similar pharmacology to ryanodine receptors and may play a general role in intracellular Ca^{2+} signalling. cADPR also stimulates Ca^{2+} release in systems as diverse as neurones[5], cardiac muscle[6], secretory cells[7,8], blood cells[9,10], cell nuclei[11] and even plant



X=N, Y=H,
cADPR (1)
X=N, Y=Br,
8-Bromo-cADPR (2)
X=N, Y=NH_2,
8-Amino-cADPR (3)
X=CH, Y=H,
7-Deaza-cADPR (4)
X=CH, Y=Br,
7-Deaza-8-bromo-cADPR (5)

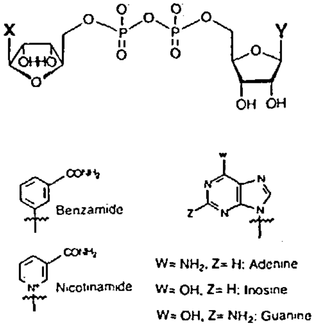
microsomes[12], suggesting that it may be a general agent. It can function as a sensitizer of CICR (Calcium Induced Calcium Release)[5] and also as a classical second messenger. cADPR may mediate glucose-induced insulin secretion[7], and can transduce abscisic acid signals in plant cells[13]. It has also been proposed to enhance Ca^{2+} entry in T-lymphocytes[9] and also to act as a second messenger in the sustained Ca^{2+} signalling of T-cells[14].

(a) Analogues of cADPR as agonists and antagonists:
We have synthesized a number of analogues of cADPR using a chemoenzymatic method[15] as agonists, antagonists and enzyme inhibitors, including the known antagonists 8-bromo (2) and 8-amino cADPR (3) and deployed them in biological analyses of cADPR-mediated Ca^{2+} release. Synthetic NAD^+ analogues are cyclised using ADP-ribosyl cyclase. The carbon-N^1-glycosidic bond is the site of breakdown of cADPR to ADP ribose. It may be that attempts to find novel activities for cADPR are being hindered by its rapid breakdown, since cADPR hydrolase activities are often significantly greater than ADP-ribosyl cyclase activities. We have therefore designed metabolically resistant analogues (e.g. a carbocyclic analogue, cyclic aristeromycin diphosphate ribose[16]). In NG-108 cells this analogue of cADPR is some 40 times more effective than cADPR at sensitizing cells to CICR[17]. 8-Substitution of the adenine ring produces a series of competitive antagonists as assessed from Ca^{2+} release assays using sea urchin homogenate or mammalian permeabilized cells[18]. A number of such analogues have been synthesised with the rank order of antagonist potency NH_2>8-Br>8-OCH_3>>8-oxy->>piperidino. Another series NH_2>$NH(CH_3)$>$N(CH_3)_2$ showed that antagonist effectiveness reduces with increasing size of molecular volume of the substituent. These compounds often behaved significantly differently in the sea urchin *vs* T-cell systems, for example 8-methoxy cADPR is very potent in T cells[14]. Modification by removing hydroxyl groups in the $2'_A$- and $3'_A$- positions results in 2'-deoxy-cADPR, a full agonist, and 3'-deoxy-cADPR, a much weaker agonist[19]. Interestingly, 3'-OCH_3-cADPR is an antagonist[19], offering a *new* avenue for the design and synthesis of such agents. Modification at the N^7 position by replacing the nitrogen with a CH group results in 7-deaza-cADPR (4), which is a partial agonist[20]. Combining modifications at the 7 and 8 positions has led to the synthesis of 7-deaza-8-bromo-cADPR (5)[21] which, as well as being non-hydrolysable and a competitive antagonist, is also membrane permeant[22]. Using 7-deaza-8-Br-cADPR, we have recently indicated a role for cADPR in mediating signalling events involved in changes in synaptic plasticity in the mammalian hippocampus[23] and as a second messenger in T-cells[14].

(b) Design of ADP-ribosyl cyclase inhibitors:
cADPR is formed via ADP-ribosyl cyclase. In an attempt to inhibit the cyclase *in vitro*, we have been using the enzyme from *Aplysia* as a model. X-Ray analysis[24], provides no mechanistic information, but hints at the probable active site. While NAD^+ is the true substrate of the *Aplysia* cyclase, nicotinamide inosine dinucleotide

(NID^+) was found to be a substrate showing interesting characteristics as a potential competitive inhibitor. NID^+ was tightly bound to the catalytic site and only slowly converted to cIDPR[25]. When we tested NID^+ against the true substrate, it behaved as a nanomolar competitive inhibitor of the cyclase. Comparison of the K_m of NAD^+ (100μM) with the K_i of NID^+ (70nM), suggested a strategy to design potent slowly reversible or even irreversible competitive inhibitors based upon inosine. To examine the validity of a proposed cyclization mechanism[25] which suggests that the active site has low recognition specificity for the purine, we have investigated the importance of each aromatic moiety of NAD^+ by synthesising bis-nicotinamide dinucleotide (Np_2N), bis-adenosine dinucleotide (Ap_2A) and bis-inosine dinucleotide (Ip_2I)[26]. Each was found to be a competitive inhibitor. However, Np_2N was a weak inhibitor, while the bis-purine nucleotides were five fold more potent[26] indicating that the purine ring is recognised by the catalytic site and the adenine is involved in NAD^+ binding.

	X	Y	K_m	K_i	K_m/K_i
NAD^+	nicotinamide	adenine	135μM		
NID^+	nicotinamide	inosine	8.80μM	70.0nM	1929
NGD^+	nicotinamide	guanine	15.5μM	143nM	944
ADPR•	OH	adenine		0.80mM	0.17
IDPR•	OH	inosine		1.30mM	0.10
nicotinamide				500μM	0.27
				1.10mM	0.12
Bp_2A	benzamide	adenine		189nM	714
Bp_2N	benzamide	nicotinamide		201nM	672
Np_2N	nicotinamide	nicotinamide		23.0μM	5.90
Ap_2A	adenine	adenine		900nM	150
Ip_2I	inosine	inosine		12.0μM	11.3



Importantly, unlike many NADases, *Aplysia* cyclase is not inhibited by ADPR or IDPR, emphasizing the differences between NADases and this cyclase. Replacement of the nicotinamide by a benzamide moiety resulted in potent inhibition at the nanomolar level[26]. These results indicate that enzymatic hydrolysis of the inhibitors could be prevented by the use of benzamide derivatives without a decrease in affinity for the cyclase active site. Our work on cADPR analogues suggests that modification of the ribose ring has only a small effect on the ability of the enzyme to cyclise the modified NAD^+. This would indicate that the binding pocket has low affinity for the adenosine ribose, which could even be envisaged as lying outside the enzyme binding pocket. However, X-ray studies on the enzyme complexed to an irreversible inhibitor should offer more interesting data on this unconventional NADase. *Aplysia* cyclase inhibitors developed here should also enable us to define leads for tackling the more relevant targets CD38 cyclase and hydrolase. Thus we have developed a number of interesting probes to investigate this new pathway of Ca^{2+} signalling.

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